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Ultrasound Enhancement of Thrombolysis and Reperfusion In Vitro

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Objectives. The aims of this study were 1) to develop an in vitro flow system in which reperfusion mediated by ultrasound-accelerated thrombolysis could be studied, and 2) to test whether ultrasound-accelerated thrombolysis could hasten reperfusion in this system.

Background. Ultrasound has been shown to increase tissue plasminogen activator (t-PA)–induced thrombolysis in vitro as assessed by radioactive fibrinogen release from labeled clots and in an animal in vivo model.

Methods. To test whether reperfusion is accelerated, we created obstructive whole blood clots in an in vitro flow system. Four control clots were exposed to ultrasound only without any thrombolytic agent (group 1). Sixteen clots were exposed to continuous infusion of recombinant tissue-type plasminogen activator rt-PA and randomized to either continuous wave ultrasound exposure at a frequency of 0.5 MHz and an intensity of 8 W/cm^2 (group 2) or to no ultrasound (group 3). Flow distal to the clot and the rate of release of radiolabeled fibrin products were used as an index of reperfusion and thrombolysis, respectively. Samples were obtained for measurements of lytic variables such as plasminogen, fibrinogen and rt-PA concentrations.

Results. Flow was significantly higher in the rt-PA–treated clots within 10 min of exposure to ultrasound than in those without such exposure (9.4 ± 9.9% of maximal flow in group 2 vs. 0.5 ± 1.5% in group 3, $p < 0.05$). The maximal difference in flow between groups 2 and 3 was achieved at 25 min (61.0 ± 30.4% vs. 14.2 ± 14.7%, $p = 0.03$). Thrombolysis was significantly higher after 15 min of ultrasound exposure (12.8 ± 9.1% in the ultrasound-treated group 2 vs. 4.0 ± 3.9% in group 3, $p < 0.05$). The maximal difference between groups 2 and 3 occurred at 25 min (26.7 ± 13.1% vs. 7.24 ± 5.7%, $p < 0.004$). Neither flow nor clot lysis occurred in group 1. Plasminogen and fibrinogen concentrations and rt-PA antigen concentrations were consistent with those observed during fibrinolytic therapy in vivo.

Conclusions. Continuous wave ultrasound at 0.5 MHz and an intensity of 8 W/cm^2 accelerates rt-PA–induced thrombolysis and reperfusion in vitro.

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Controlled clinical trials have shown the efficacy of thrombolytic therapy in reducing mortality (1,2) and in preserving left ventricular function (3–5). Because the amount of myocardial muscle loss is proportional to the duration of ischemia (6–8), efforts are currently being directed to enhancing the rate of thrombolysis by the use of adjuvant therapy (9).

Ultrasound at low frequencies (20 kHz) and high intensities accelerates clot disruption in vitro (10–13) and in a canine model using either pulsed (10,12) or continuous wave ultrasound. Such ultrasound was delivered by catheter directly to the clot, and no lytic therapy was used. Other preliminary studies have shown that catheter-delivered ultrasound can enhance thrombolysis in vitro, using urokinase (14) and recombinant tissue-type plasminogen activator (rt-PA) (15).

A qualitatively different approach has been taken by Kudo (16), who delivered ultrasound at lower intensities and much higher frequencies noninvasively in an in vivo system in which rt-PA was used. We (17) have shown that ultrasound at similar frequencies (1 MHz) and intensities lower than those of the catheter-based ultrasound system (14,15) can accelerate thrombolysis mediated by rt-PA in an in vitro system of test tubes (17). Lauer et al. (18) showed that ultrasound can enhance rt-PA–induced thrombolysis in vitro and in vivo although the enhancement in the rabbit jugular vein thrombosis model was not statistically significant.

The aims of this study were 1) to develop an in vitro flow system in which reperfusion mediated by ultrasound-accelerated thrombolysis could be studied, and 2) to test whether ultrasound-accelerated thrombolysis could hasten reperfusion in this system.

Methods

Clot preparation. Human fibrinogen (Grade L, Helena Laboratories) was radiolabeled with iodine-125, using the iodogen technique (19). The labeled fibrinogen was 95% clottable. An aliquot of 5 to 7 μliters of radiolabeled fibrinogen was added to 500 μliters of the citrated fresh blood obtained from two normal volunteers. The total radioactivity of the blood was measured in a gamma counter. Fifty μliters

From the Cardiology and Hematology Units and the Center for Biomedical Ultrasound, University of Rochester, Rochester, New York. Genentech Inc., San Francisco, California supplied the recombinant tissue-type plasminogen activator (rt-PA) for this study.

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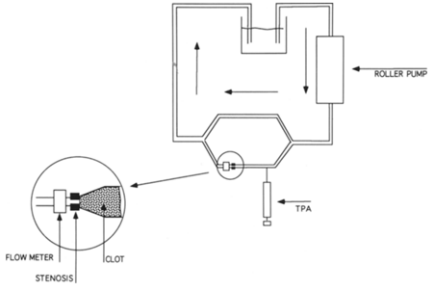

Figure 1. Diagram of the flow system. **Upper middle,** A beaker containing plasma serves as a reservoir. Plasma continuously circulates by a roller pump in the tube system. One branch of the system is completely occluded by a radiolabeled clot situated proximal to a 1.2 mm in diameter narrowing. Recombinant tissue-type plasminogen activator (tPA) is continuously infused immediately proximal to the clot. A flow probe measures the rate of flow distal to the clot. The three **unlabeled arrows** point in the direction of flow.

of 2 mol/liter calcium chloride was added and then transferred to a conical polyethylene tube, which made part of the tube system just proximal to the point of maximal narrowing (see Fig. 1 and flow system section below). Five units of bovine thrombin (Calbiochem Corp) dissolved in 20 μliters of buffer solution (see later) were added. Clots were left in a vertical position for 1 h at room temperature to permit retraction.

Flow system. A closed circuit flow system was developed (Fig. 1). The circuit was made of plastic tubes (Tygon, Norton Industrial Plastics). The two ends of the circuit were immersed in a beaker containing fresh-frozen plasma obtained through the American Red Cross (Rochester Region). The tube system had a bifurcation to two parallel branches. A polyethylene conical tube (Fisher Scientific) in which the clot was formed was part of one of the two branches. The tip of the polyethylene tube was connected to a plastic connector with an internal diameter of 1.2 mm that formed the narrowest part of the circuit. An in-line ultrasound flow probe connected to a flow meter (Transonic Systems) was located just distal to the point of maximal narrowing. The flow probe resolution was 0.1 ml/min and it was calibrated for blood at 37°C. The rt-PA was continuously injected from a side arm, just proximal to the clot. A roller pump (Masterflex) operated at a constant velocity provided flow in the system.

Ultrasound. Clots were located at the focus of the ultrasound field in a tank containing distilled water at 37°C. The source of ultrasound was a flat, 2.54-cm diameter, cylindric piezoelectric transducer driven at 0.5 MHz in the continuous wave mode. A 3-cm thick block of rubber was used as an acoustic absorber behind the clot to limit ultrasound reflection and standing waves. The clots were placed 5.2 cm from the source of ultrasound where the 3-dB ultrasound beam width was 6 mm. The spatial peak intensity at the focus was 8 W/cm^2, measured by a needle-type hydrophone with a 1.0-mm active element (model NP-1000, NTR Systems) that was calibrated against a spheric steel radiometer (20). Transmission loss through the polyethylene tube that housed the clot was 0.1 dB at 0.5 MHz.

Four clots were exposed to ultrasound only without any thrombolytic agent (group 1). Other clots had continuous infusion of rt-PA immediately proximal to them, and were randomized to either ultrasound exposure (group 2) or to no ultrasound (group 3). Except for group 1 studies, the operator who handled the sampling and analysis did not know whether the ultrasound system was in use until after the data analysis had been completed.

Experimental protocol. Retracted fresh whole blood clots were put in one branch of a flow system that was filled with 200-ml heparinized (1 U/ml) fresh-frozen plasma (heparin from Riker Lab., Inc.). Flow was continuously monitored, and flow readings were obtained every 5 min for 2 h. At the end of the experiment the residual clot was removed and the maximal flow measured. Flow readings were expressed as a percent of maximal flow. Experiments with an initial (pre rt-PA infusion) flow reading of ≥5% of maximal flow were excluded from analysis because full obstruction was not obtained. Two milligrams of rt-PA (Genentech) was dissolved in 30 ml of buffer solution. An initial bolus of 3 ml of the rt-PA solution (0.2 mg) was injected at a constant rate for 3 min; then 1.8 mg was continuously injected at a constant rate of 0.02 mg/min (0.3 ml/min) for 90 min. Injection was performed using a Harvard Apparatus infusion pump. Plasma samples of 200 μliter were obtained from the beaker every 5 min for measurements of radioactivity. Because mixing of the labeled fibrin products in the beaker was homogeneous, this procedure provides data needed to analyze the rate of thrombolysis, which is related to the rate of radioactive fibrin release from the clot. Therefore, the recovery of labeled fibrin products in this system is flow dependent. Samples were obtained as duplicates, and the mean radioactive reading of the two samples was used for analysis. Assuming a volume of distribution of 210 ml, the radiolabeled fibrin counting was calculated to percent of clot

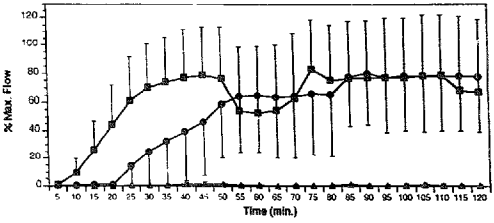

Figure 2. Percent of maximal flow distal to the clot (mean ± 1 SD) plotted against time. Group 1 (**triangles:** control ultrasound only, no recombinant tissue-type plasminogen activator [rt-PA]); group 2 (**squares:** ultrasound and rt-PA), and group 3 (**circles:** rt-PA only, no ultrasound).

lysis, when the initial counting of the labeled blood served as 100%.

The initial length of the clot was 2.5 to 3 cm, which is longer than the focal zone of the ultrasound beam. Therefore the proximal two thirds of the clot (the side nearest the rt-PA infusion site) was moved along the ultrasound beam several times during the ultrasound exposure, as the proximal portion dissolved. However, the distal portion of the clot was not exposed to the ultrasound beam.

rt-PA, fibrinogen and plasminogen measurements. Samples of 1 ml of fresh-frozen plasma were collected at baseline, after 15 min and at the end of the experiments for plasminogen and fibrinogen measurements. Samples of 200 μliter for t-PA antigen concentration measurements were obtained at baseline, immediately after bolus injection and at 15, 60, 90 min and at the end of the experiments. All samples were collected in duplicate. The samples were kept frozen at −20°C and were analyzed together at the end of the experiments.

Fibrinogen was measured using a modification of the method of Von Clauss (21). Plasminogen was assayed using the end point method (22). Values are expressed as percent of the concentration in pooled normal plasma. The t-PA antigen concentration was determined using an ELISA (Imubind-5, American Diagnostica Inc.).

The results of the hematologic tests were expressed as mean value ± 1 SD.

Microscopic analysis. At the end of the experiments, samples were withdrawn from the beaker and observed under a light microscope (Bausch & Lomb) at a high power field (×400) for particulate size analysis and to characterize debris shape and composition. Plasma was examined for the presence of fibrin fragments, clusters of red blood cells and red blood cell morphology. Particles were measured using a calibrated eyepiece micrometer and hemocytometer.

Statistical analysis. The mean value ± SD of the percent clot lysis and the percent maximal flow rate were calculated at each time interval and results are reported as mean value ± 1 SD. The statistical difference among means of the three groups was calculated using one-factor analysis of variance (ANOVA). The statistical difference between means of each pair of treatments, at 5-min time intervals, was tested using a two-tailed Student *t* test for unpaired data with adjustment made using the Bonferroni correction. Differences were considered significant at $p < 0.05$.

Results

Flow and clot lysis. Reperfusion did not occur (Fig. 2) and clot lysis approximated zero (Fig. 3) in four experiments using ultrasound without rt-PA (group 1). Reperfusion was obtained in 14 of the 16 experiments using rt-PA and randomized to either ultrasound (group 2) or no ultrasound (group 3). In one experiment in each group no reperfusion

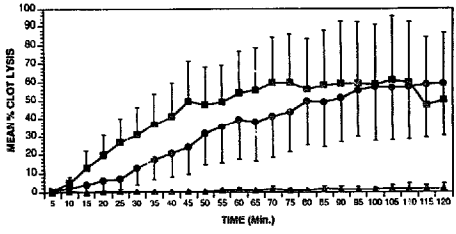

Figure 3. Percent clot lysis (mean value ± 1 SD) plotted against time. Group 1 (**triangles:** control ultrasound only, no recombinant tissue-type plasminogen activator [rt-PA]), group 2 (**squares:** ultrasound and rt-PA), and group 3 (**circles:** rt-PA only, no ultrasound).

occurred and clot lysis as measured by measurements of radiolabeled fibrin products in the beaker was near zero.

Continuous wave ultrasound at 8 W/cm^2 enhanced thrombolysis by rt-PA as measured either by flow or by direct measurement of clot lysis in vitro using release of radiolabeled fibrin. A trend toward ultrasound-enhanced clot lysis (Fig. 3) was noted after 10 min (4.9 ± 3.3% in group 2 vs. 2.2 ± 2.0% in group 3). After 15 min this reached statistical significance (12.8 ± 9.1% in group 2 vs. 4.0 ± 3.9% in group 3, $p < 0.05$). The maximal statistical significance was obtained at 25 min (26.7 ± 13.1% in group 2 vs. 7.24 ± 5.7% in group 3, $p < 0.004$). Restoration of flow (Fig. 2) was significantly earlier and the flow was significantly higher after 10 min in group 2 (with ultrasound exposure) than in group 3 (9.4 ± 9.9% vs. 0.5 ± 1.5% of maximal flow, $p < 0.05$).

Ultrasound exposure resulted in a marked shortening of the time interval required to exceed >50% of maximal flow (Fig. 2): 61.0 ± 30.4% of maximal flow was achieved after 25 min of ultrasound exposure in group 2 compared with 58.8 ± 38.7% after 50 min of rt-PA infusion to the nonexposed clots of group 3. Ultrasound exposure also shortened the time to obtain at least 50% of clot lysis (Fig. 3): 54 ± 22.7% of clot lysis was obtained after 60 min of exposure to ultrasound in group 2 compared with 90 min of rt-PA infusion required to achieve 51.3 ± 24.5% of clot lysis in the nonexposed clots of group 3.

The mean percent clot lysis was higher in group 2 (with ultrasound) than in group 3. This difference was statistically significant for up to 45 min, when 49.4 ± 22.0% of the clot was lysed in group 2 compared with 24.0 ± 14.3% in group 3 ($p < 0.04$).

At 55 min, the mean flow rate of group 2 was lower than that in group 3 (53.8 ± 44.4 vs. 64.2 ± 39.9% of maximal flow, $p = 0.63$). This is illustrated as a dip in the upper curve in Figure 2. This difference lasted for 20 min, when the mean flow rates almost equalized in both systems. Visual observation made in the transparent tube system showed that in three experiments with ultrasound and in one without ultrasound, a small portion of clot was detached from its distal end, which was not exposed to ultrasound, and advanced into the narrowest part of the circuit, causing reocclusion. This remnant was totally lysed by the continuous infusion of rt-PA, causing restoration of flow within 15 to 20 min.

rt-PA, fibrinogen and plasminogen measurements. Table 1 represents the mean concentration of plasminogen and fibrinogen achieved during six of the experiments in which rt-PA was infused. These levels were significantly reduced from the baseline concentration as the rt-PA infusion proceeded. The tPA antigen concentration increased with continuous drug infusion and no clearance.

Microscopic analysis. To determine if the clot was disrupted into large fragments by ultrasound, the plasma in the beaker reservoir was carefully observed at the end of most experiments. No macroscopic fragments could be seen. Samples taken from the beaker after two experiments were also observed by light microscopy. No clot fragments, clusters of red blood cells or fibrin fragments could be seen, and the red blood cell morphology was normal.

Table 1. Fibrinolytic Data

Time (min)	Plasminogen (%)	Fibrinogen (mg/dl)	t-PA Antigen (ng/ml)
0	101.8 ± 36.4	385.2 ± 61.6	3.66 ± 2.1
3	—	—	778 ± 462.1
15	62 ± 24.1	185.2 ± 41.9	1,880 ± 1092.6
60	—	—	2,330 ± 922.2
90	—	—	2,990 ± 520.1
120	21.2 ± 4.1	116.4 ± 27.3	3,240 ± 622.9

The concentration of plasminogen is expressed as percent of the concentration in pooled normal plasma. The concentration of fibrinogen is expressed as mg/dl of plasma, and the concentration of tissue plasminogen activator (t-PA) antigen is expressed as ng/ml of plasma. All data are given as mean value ± 1 SD. — = measurement not made.

Discussion

This study demonstrates that 0.5-MHz continuous wave ultrasound at a peak intensity of 8 W/cm^2 accelerates thrombolysis of fresh whole blood clots induced by rt-PA. The ultrasound effect was obtained rapidly, and a significant acceleration of thrombolysis was observed at 15 min by both restoration of flow and by radiolabeled fibrin product measurements. This significant enhancement of thrombolysis was sustained for 35 to 45 min in this experimental model.

The effect of ultrasound described in this report differs from that in several previously reported studies (10,12,13) in which ultrasound was delivered by catheter at much lower frequencies (20 kHz) and at higher intensities. Therefore, ultrasound catheter systems create violent cavitation and local large amplitude stirring, producing fragments of variable size. Such an invasive procedure was applied in vivo in a canine model of femoral artery or vein thrombosis (10,12,23,24). Using a noncatheter approach, Rosenschein et al. (25) reported thrombus ablation using shock waves of high power acoustic energy. A potential explanation for the lack of effect of ultrasound alone in the current experiment compared with that in these prior studies is that the ultrasound in the current study was of higher frequency and lower intensity.

The clots in the present study were 2.5 to 3 cm long. This dimension was several times the ultrasound beam width of 6 mm at the focus, where the clots were exposed. It is potentially important that ultrasound exposure of only part of the full thrombus can result in accelerated thrombolysis and reperfusion. The most distal portion of the clot was separated from the port of infusion of the lytic drug by the long clot interposed between them.

One might observe that the initial benefit obtained by enhancement of clot lysis was not sustained, and the curves of the two treatment groups plotted against time actually converged after about 80 min. This finding could be ex-

plained by the lack of drug clearance in this in vitro system. Thus, the drug reached a very high concentration and eventually induced complete lysis. No difference could be observed between the two treatment groups after 85 to 90 min, because all clots were entirely lysed and the tubing reperfused.

Ultrasound can cause biologic effects through several mechanisms (26,27) such as heating, acoustic cavitation and microstreaming. We speculate that the mechanism of ultrasound enhancement of thrombolysis may be enhancement of transport of the lytic agent to the clot by an acoustic effect not yet understood; however, it was the purpose of this study to investigate efficacy rather than to determine mechanism.

Limitations of the study. Although plasminogen and fibrinogen concentrations and tPA antigenicity were found to be consistent with those observed during fibrinolytic therapy in vivo, an in vitro system does not entirely mimic in vivo thrombolysis and reperfusion in all its complexities, including clotting and thrombolytic physiology as well as vascular anatomy, physiology and ultrasound exposure variables. However, we believed that the simplified system described in this study might be useful to study efficacy and various relevant ultrasound exposure variables.

The assay for fibrinolysis, measurement of soluble radiolabeled products in the beaker reservoir, was flow dependent. Partial clot lysis could occur without appearance of the fibrin degradation products in the beaker if a fully obstructive clot was present. Thus, clot lysis appeared to increase approximately 5 min after the improvement in flow. However, total obstruction did not remain throughout the majority of experiments. Reperfusion was obtained in seven of eight experiments in group 2 (with ultrasound) and in seven of eight experiments in group 3 (without ultrasound) but in none of the group 1 control experiments without rt-PA.

Conclusions. We conclude that ultrasound can accelerate rt-PA–induced thrombolysis and reperfusion in vitro.

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